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INVESTIGATION OF MRP-1 PROTEIN AND MDR-1 P-GLYCOPROTEIN EXPRESSION IN INVASIVE BREAST CANCER: A PROGNOSTIC STUDY

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The efficacy of breast cancer treatment is limited by the development of resistance to various chemotherapeutic agents. We conducted a retrospective study of the expression of 2 drug resistance efflux pumps, MRP-1 and MDR-1 Pgp, in 177 invasive breast carcinomas. Immunohistochemical expression of these proteins was correlated with clinicopathologic characteristics as well as relapse-free survival (RFS) and overall survival (OS) times. MDR-1 Pgp was associated strongly with higher histologic grade (grade III). A highly significant association was shown between MDR-1 Pgp and MRP-1 expression ($p < 0.01$), 47.4% of patients expressing both proteins; MRP-1 was expressed in approximately 61% of patients and MDR-1, in approximately 66% of patients. No association was shown in the overall group between either MDR-1 Pgp or MRP-1 and any of the other clinicopathologic features. Kaplan-Meier analysis revealed that in a subset of patients with either high-grade (grade III) stage 1 (node-negative) or stage 2 (node-positive) tumours who were treated with surgery followed by adjuvant chemotherapy, MRP-1 expression in <25% of tumour cells at diagnosis was significantly associated with improved RFS ($p < 0.02$) and OS ($p < 0.02$). Using multivariate analysis, MRP-1 expression in <25% of tumour cells at diagnosis was identified as an independent, significant prognostic factor for RFS ($p < 0.01$) and OS ($p < 0.01$) in this patient group but not in other groups. In this subgroup, no significant correlation was observed between expression of MDR-1 Pgp and MRP-1. While the number of patients with high-grade tumours treated with adjuvant chemotherapy was small and further confirmatory research is warranted, it appears that assessment of MRP-1 expression at diagnosis may offer useful prognostic information in subgroups of patients with stage 1 or stage 2 high-grade tumours who receive CMF-based adjuvant chemotherapy. Given the known substrate specificities of MRP-1, any mechanistic relationship between MRP-1 expression and CMF resistance remains unclear. No association was shown between MDR-1 Pgp expression and either RFS or OS time in any subgroup of patients.

Key words: *breast cancer; MRP-1; MDR-1; Pgp; immunohistochemistry; prognosis; relapse-free survival; overall survival*

Breast cancer is the most common cause of cancer death in the female population in the Western world.[1] Management of breast carcinoma has changed considerably over the past 20 years. The number of available therapeutic options has widened considerably, and there is an increasing range of hormonal, cytotoxic and more recently MAb-targeted drug regimes available for both the adjuvant and neoadjuvant settings.[2] However, despite the advances in both detection and treatment, 40–50% of patients diagnosed will eventually die of the disease.[3] It is thus vital to determine the optimal treatment modality for each individual patient and to identify subgroups of patients who might benefit from individualised treatment strategies. Identification of biologic markers which might predict clinical outcome (prognostic markers) and the likelihood of a response to a particular type of adjuvant therapy (predictive markers) will facilitate this.

Treatment of breast cancer patients with operable disease is stage-dependent. Stage 2 patients (those with involvement of axillary nodes) have a 10-year average survival of <50% and will receive adjuvant chemotherapy following surgery.[4] Stage 1 patients (node-negative) are cured by surgery in 70% of cases. Because of the 30% risk of relapse, many of these patients will also be treated with chemotherapy or hormonal therapy.

The emergence of drug resistance is one of the main obstacles to successful chemotherapy in this disease. MDR is a phenomenon whereby tumour cells acquire resistance to a broad range of structurally and functionally diverse chemotherapeutic drugs, including anthracyclines, vinca alkaloids, epipophyllotoxins and paclitaxel (Taxol), following exposure to a single agent.[5]

Most attention has been directed to the role played by membrane transporter proteins belonging to the ATP binding cassette superfamily in MDR observed in breast cancer, particularly by the MDR-1 Pgp drug transporter[6] and another efflux pump, MRP-1, which encodes a 190 kDa membrane-bound glycoprotein.[7] Since the initial discovery of MRP-1, additional members of this family have been described (MRPs 2–8).[8–11] The specific role of these proteins in clinical drug resistance has not been fully elucidated.

Studies have reported conflicting results with regard to the prognostic and/or predictive role of MDR-1 Pgp in breast cancer. MDR-1 Pgp is expressed in approximately 41% of untreated breast cancers, and prior exposure to chemotherapy increases this expression.[12] There is some evidence supporting a correlation between MDR-1 Pgp expression at diagnosis and long-term outcome in both the adjuvant and neoadjuvant settings.[13] Some studies have suggested that evaluation of MDR-1 Pgp expression following

Abbreviations: ABC, avidin–biotin complex; BCRP, breast cancer resistance protein; CMF, cyclophosphamide, methotrexate and 5-fluorouracil; DAB, 3,3′-diaminobenzidine; DCIS, ductal carcinoma *in situ;* DPX, dextropropoxyphene; ER, oestrogen receptor; HRP, horseradish peroxidase; LN, lymph node; MAb, monoclonal antibody; MDR-1, multiple drug resistance protein-1; MRP-1, multiple drug resistance–associated protein-1; OS, overall survival; Pgp, P-glycoprotein; PR, progesterone receptor; RFS, relapse-free survival; TBS, TRIS-buffered saline.

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TABLE I – CLINICOPATHOLOGIC FEATURES OF PATIENTS STUDIED FOR MDR-1 Pgp AND MRP-1 EXPRESSION

	MDR-1 Pgp	MRP-1
Age range (years)	31–90	31–90
<50	53 (30%)	52 (30%)
>50	124 (70%)	122 (77%)
Metastases to axillary lymph nodes (stage 2)	95 (53.6%)	93 (53.4%)
Negative nodal status (stage 1)	82 (46.3%)	81 (46.6%)
ER-negative	56 (31.6%)	55 (31.6%)
ER-positive	113 (63.8%)	111 (63%)
ER status unknown	8 (4.6%)	8 (5.4%)
Tumour size range	0.6–9.0 cm (mean 2.88 cm)	0.6–8.0 cm (mean 2.83cm)
<2 cm (T1)	33 (18.6%)	33 (19%)
2–5 cm (T2)	135 (76.3%)	133 (76.5%)
>5 cm (T3)	9 (5%)	8 (4.6%)
Histologic grade		
I	20 (11.3%)	19 (10.9%)
II	75 (42.2%)	73 (42%)
III	82 (46.3%)	82 (47.1%)
Histologic subtype		
Infiltrating ductal	143 (81%)	141 (81%)
Infiltrating lobular	26 (15%)	26 (14.9%)
Infiltrating mucinous/tubular	8 (4%)	7 (4%)
Adjuvant chemotherapy	90 (50.8%)	86 (49.9%)
Node-positive patients	61 (64.2%)	60 (64.5%)
Node-negative patients	28 (34.1%)	26 (32%)
None	73 (41.2%)	73 (42%)
Chemotherapy details unknown	15 (8%; 9 node-negative, 6 node-positive)	9 (9%; 4 node-negative, 5 node-positive)
Postoperative tamoxifen treatment	108 (61%)	106 (61%)
Node-positive patients	58 (61%)	58 (62.3%)
Node-negative patients	62 (76%)	48 (59.3)
None	4 (30.5%)	53 (30.5%)
Tamoxifen details unknown	16 (8.5%; 6 node-negative, 10 node-positive)	15 (8.5%; 6 node-negative, 9 node-positive)

chemotherapy may offer more prognostic value than its expression prior to treatment.[14]

MRP-1 is expressed by a considerable number of untreated breast tumours (on average 49%).[12,13] In primary breast cancers, MRP-1 expression is inversely correlated with both RFS and OS.[15,16] MRP-1 expression at diagnosis is predictive of OS in patients who received adjuvant systemic chemotherapy with CMF, in patients with small tumours (T1) and in node-negative patients.[16] It is also an important predictor of poor prognosis in patients with recurrent breast cancer.[17] Therefore, MRP-1 expression at diagnosis may be associated with a worse prognosis in breast cancer. As in the case of MDR-1 Pgp, it is more difficult to define the exact role of MRP-1 in the clinical drug resistance observed in this disease.

To address the prognostic or possible predictive role of MRP-1 and/or MDR-1 Pgp expression in invasive breast cancer, we determined the frequency of the expression of these 2 proteins in stage 1 and stage 2 invasive breast cancers, treated by surgery with or without adjuvant chemotherapy and/or hormonal therapy, with a minimum 6 years of follow-up. Expression of these proteins was correlated with known clinicopathologic features and with RFS and OS.

MATERIAL AND METHODS

Patients

The patient group comprised women diagnosed with primary tumours of the breast: 177 were analysed for MDR-1 Pgp expression and 174 of these, for MRP-1 expression. All patients were treated with surgery including axillary node dissection at St. Vincent's University Hospital in 1993–1994. Pathologic material was examined for each case (S.K.). Tumours were typed as described by Page and Anderson[18] and graded as described by Elston and Ellis.[19] Formalin-fixed, paraffin-embedded material was available for all patients. Representative 5 μm sections of tissue blocks were cut using a microtome, mounted onto poly-l-lysine-coated slides and dried overnight at 37°C. Slides were stored at room temperature until required. Relevant clinicopathologic features, treatment and follow-up were compiled for all 177 patients.

Immunohistochemistry

All immunohistochemical studies were performed according to the method of Hsu *et al.*,[20] using an ABC HRP-conjugated kit plus an appropriate secondary antibody. Tissue sections were dewaxed in xylene (2 × 5 min), rehydrated in graded alcohols and placed in TBS/0.05% (v/v) Tween-20. Endogenous peroxidase activity was quenched by placing tissue sections in 3% (v/v) H_2O_2/distilled H_2O for 5 min. All slides were blocked for nonspecific staining with 20% (v/v) normal rabbit serum for 20 min. Primary antibodies were applied to each sample optimally diluted in TBS/0.1% (v/v) Tween-20 [anti-MDR-1, clone 6/1C (National Institute for Cellular Biotechnology),[21] ascites diluted 1/30 to 1/50; anti-MRP-1 MAb, MRP-R1 (Alexis, Nottingham, UK),[22] diluted 1/25]. Primary antibodies were incubated overnight at 4°C or for 2 hr at room temperature, followed by 30 min incubation with biotinylated rabbit antimouse IgG [1/300 dilution in TBS/0.1% (v/v) Tween-20; E0354 from Dako, High Wycombe, UK] for detection of MDR-1 or biotinylated rabbit antirat 1gG [1/500 dilution in TBS/0.1% (v/v) Tween-20; E0468 from Dako] for detection of MRP-1. Finally, Vectastain Elite ABC reagent (HRP-conjugated; PK-7100 from Vector, Peterborough, UK) was applied using the peroxidase substrate DAB as a chromogen. All tissue sections were lightly stained with Crazzi's haematoxylin. Following dehydration in graded alcohols, slides were cleared in xylene and mounted in DPX (BDH, Poole, UK). Normal bronchial and placental tissues were used as positive controls for MRP-1, and normal kidney was used as a positive control for MDR-1 Pgp. Negative control slides, in which sections were immunostained as outlined but primary antibody was omitted, were included in all experiments.

Immunohistochemical scoring

MRP-1 and MDR-1 Pgp immunohistochemical staining was evaluated semiquantitatively (by S.K. and R.P.), according to the percentage of cells showing specific immunoreactivity and the

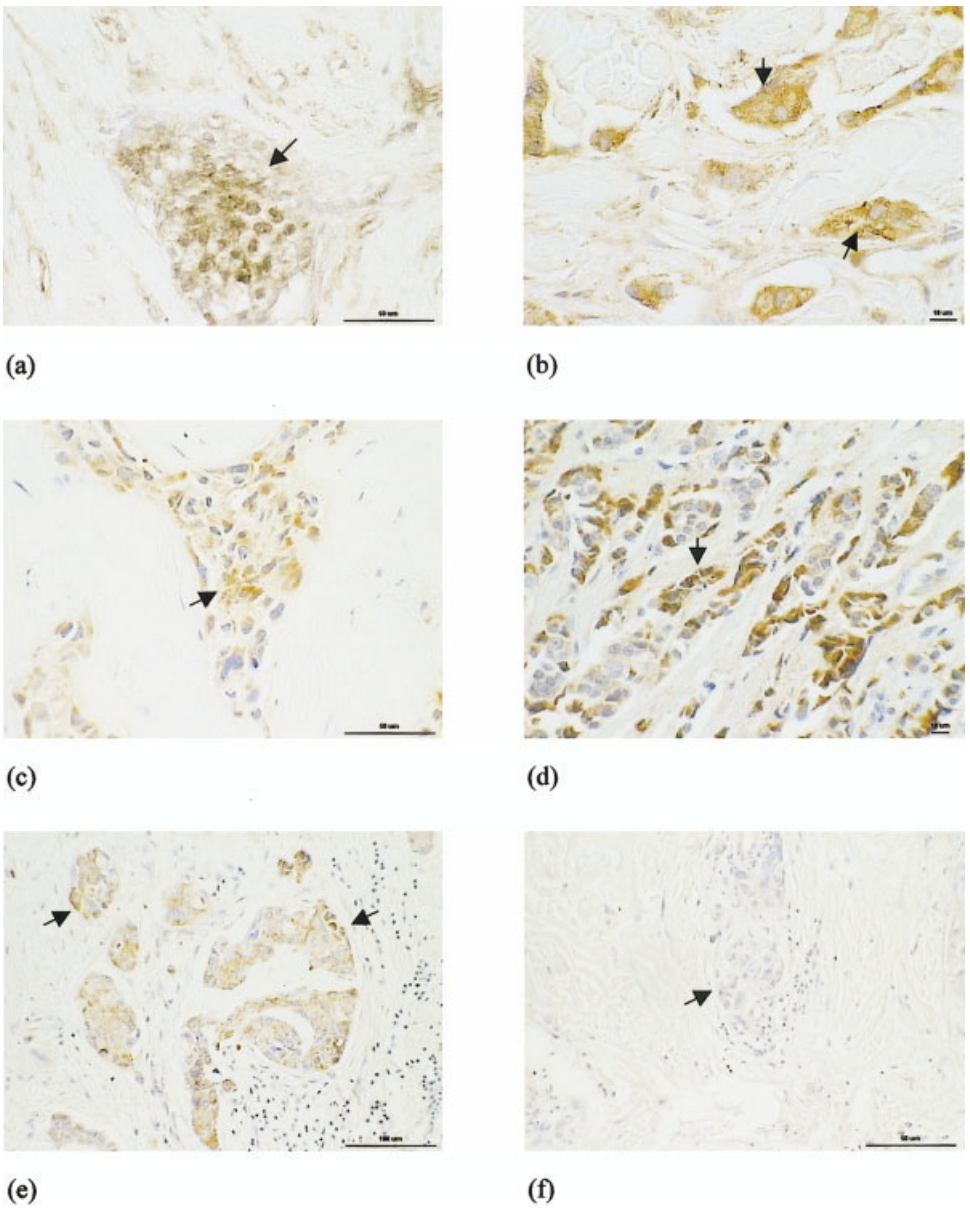

FIGURE 1 – Immunohistochemical analysis of MRP-1 protein and Pgp in invasive breast carcinomas. *(a,b)* Pretreatment tumours from invasive carcinoma patients stained with anti-MDR-1 clone 6/1C. Strong MDR-1 Pgp positivity can be observed in infiltrating ductal carcinomas in both tumour samples. *(a)* Scale bar = 50 μm, original magnification ×40; *(b)* scale bar = 10 μm, original magnification ×60. *(c–e)* Pretreatment invasive carcinomas stained with MRP-R1 MAb; intense MRP-1-positive staining can be observed in infiltrating tumour cells *(c,d)* and in both DCIS component and infiltrating tumour cells *(e)*. *(c)* Scale bar = 50 μm, original magnification ×40; *(d)* scale bar = 10 μm, original magnification ×40; *(e)* scale bar = 100 μm, original magnification ×20. *(f)* Negative control tissue pretreatment invasive carcinoma, where no staining is observed in tumour cells. Scale bar = 50 μm, original magnification ×40. All sections were counterstained with haematoxylin.

intensity of this immunoreactivity. In the case of MRP-1 and MDR-1 Pgp, membrane and cytoplasmic staining was scored as positive or negative. Semiquantitative scores were as follows: Assessment of overall positivity: 0, no tumour cells showing positive MRP-1/MDR-1 Pgp positive staining; 1+, 1–24% of tumour cells showing positive MRP-1/MDR-1 Pgp positive staining; 2+, 25–50% of tumour cells showing positive MRP-1/MDR-1 Pgp positive staining; 3+ >50, >50% of tumour cells showing positive MRP-1/MDR-1 Pgp positive staining. Intensity of MRP-1/MDR-1 Pgp staining; 1 = weak; 2 = moderate; 3 = strong.

Statistical analysis

Statistical analyses were performed using the SPSS (Chicago, IL) 10.1 software package. Descriptive statistics were used to summarise patient characteristics. The association of MRP-1 and MDR-1 Pgp with various clinicopathologic features was evaluated using the χ^2 test. Survival analyses (RFS and OS) were performed using the Kaplan-Meier method; differences between survival curves were analysed using the log rank test. Multivariate survival analyses were performed using Cox's proportional hazards model (backward stepwise, likelihood ratio). For all tests, $p < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics are described in Table I.

Immunohistochemical analysis

MRP-1 expression. MRP-1-specific staining (cytoplasmic and membranous positivity, which was granular in nature in some cases) was observed in 61.5% (107) of tumours analysed overall. Representative MRP-1-positive tumours can be observed in Figure 1*c* (score of 1+2), 1*d* (score of 3+3) and 1*e* (score of 3+2), where MRP-1-positive staining can be seen in both infiltrating cells and DCIS. Patchy, variable MRP-1 expression was observed in some DCIS cases. MRP-1 protein expression was not observed in 38.5% (67) of tumours. No MRP-1 positivity was found in benign lobules, and focal expression was seen in the majority of large ducts and lactiferous sinuses.

MDR-1 Pgp expression. MDR-1 Pgp-specific staining was observed in 66.1% (117) of tumours analysed overall. Representative

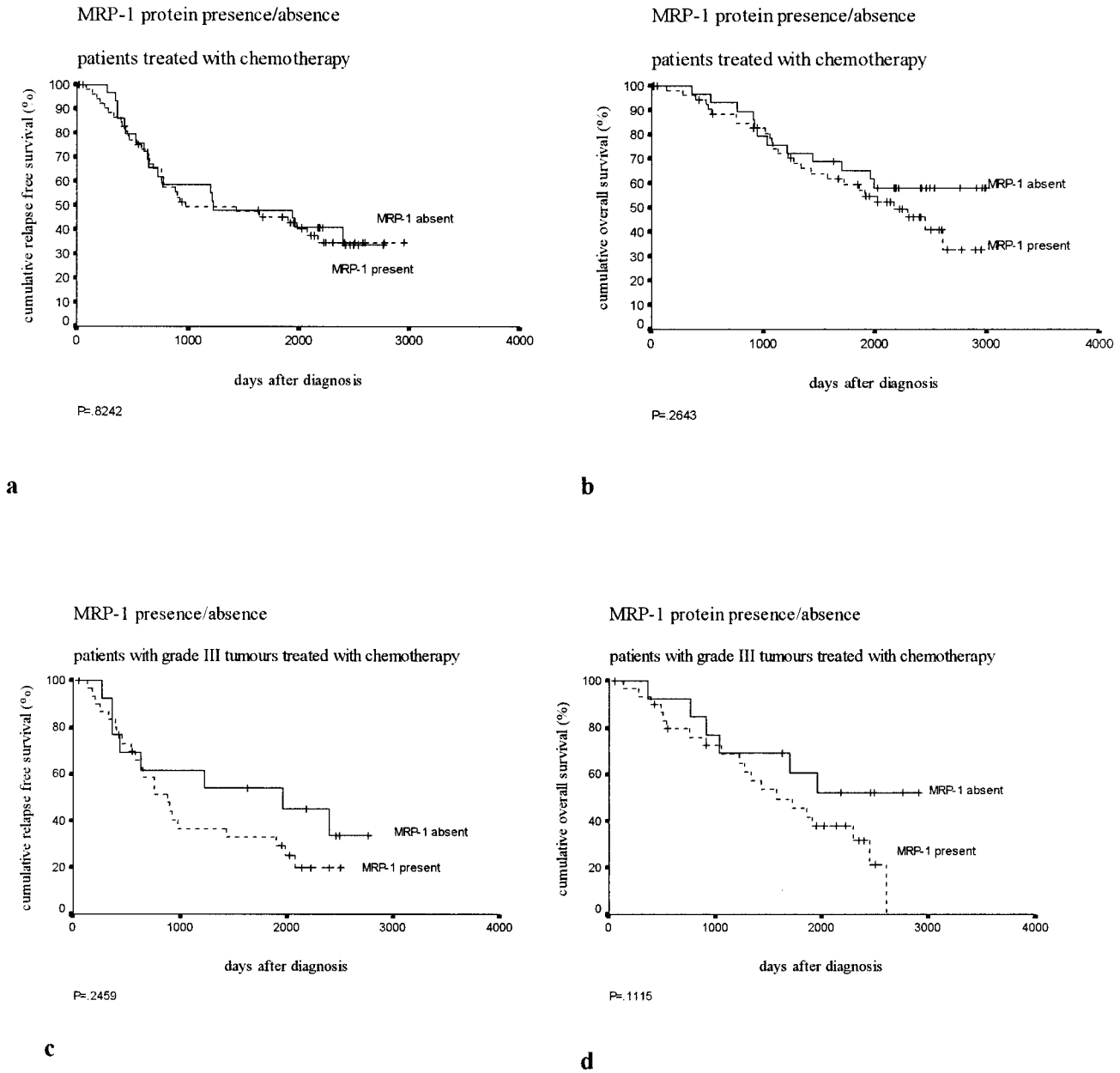

FIGURE 2 – Kaplan-Meier survival analysis for presence/absence of MRP-1 protein in all patients who received chemotherapy *(a,b)* and in chemotherapy-treated patients with grade III tumours *(c,d)* for RFS and OS, respectively. No association can be shown between presence/absence of MRP-1 expression at diagnosis and either RFS or OS in either group (p = log rank value).

MDR-1-positive tumours can be observed in Figure 1*a,b* (scores of 2+3 and 3+3, respectively), where MDR-1 Pgp-positive staining can be seen in infiltrating tumour cells. Specific staining was localised to the inner plasma membrane/cytoplasm. Staining of DCIS was also observed in some cases. MDR-1 Pgp expression was not observed in 33.9% (60) of tumours.

Correlation between MRP-1 and MDR-1 Pgp expression. Using χ^2 analysis, a highly significant correlation ($p < 0.0001$) was shown between the expression of these 2 proteins: 82 (47.4%) patients showed coexpression of the 2 proteins, while 35 (20.2%) patients did not express either protein. When patients were stratified into further subgroups, a highly significant correlation was also observed between MRP-1 and MDR-1 Pgp in patients who received chemotherapy ($p = 0.001$); 44/87 (50.6%) of these patients expressed both proteins, while 18/87 (20.7%) were negative for both MDR-1 Pgp and MRP-1 expression. No correlation was shown between these 2 proteins in patients with grade III tumours that were treated with chemotherapy ($p = 0.815$).

MRP-1 expression and relation to clinicopathologic features. Using χ^2 analysis, MRP-1 expression at diagnosis was significantly associated with subsequent tamoxifen treatment ($p = 0.033$). Expression of this protein was not significantly associated with other clinicopathologic features, *i.e.,* histologic grade ($p = 0.378$), subtype ($p = 0.652$), tumour size ($p = 0.957$), LN status ($p = 0.151$), ER status ($p = 0.630$) or adjuvant chemotherapy ($p = 0.679$).

MRP-1 expression in relation to RFS and OS. MRP-1 expression at diagnosis was analysed in relation to RFS and OS by Kaplan-Meier survival analysis, to establish any prognostic role

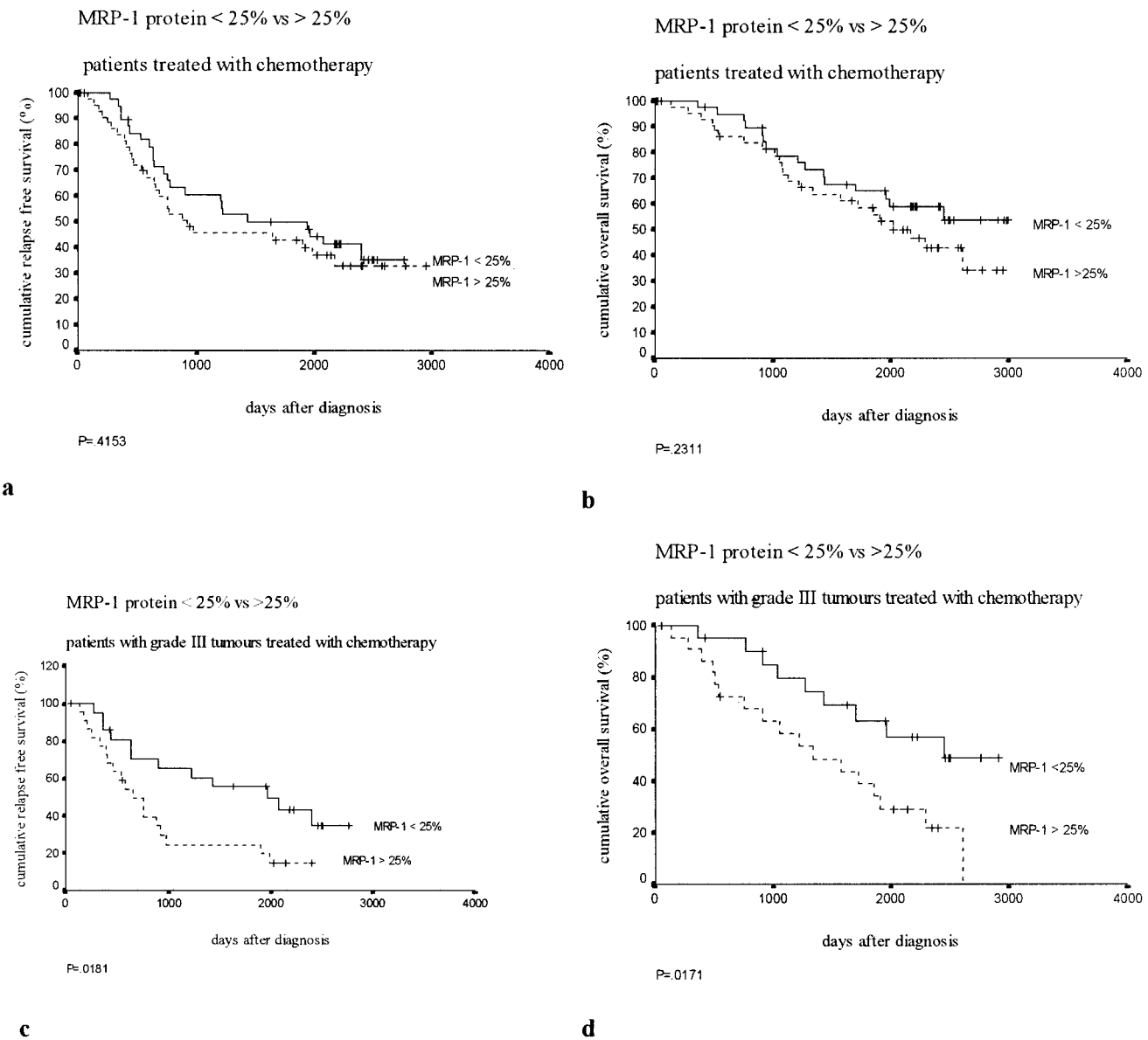

FIGURE 3 – Kaplan-Meier survival analysis for MRP-1 protein expression (<25% *vs.* ≥25% tumour cells staining positive) in all patients who received chemotherapy *(a,b)* and patients with grade III tumours treated with chemotherapy *(c,d)*. A significant association can be seen between MRP-1 expression in <25% tumour cells and improved RFS and OS in patients with grade III tumours *(c,d)* (p = log rank value).

for expression of this protein. When Kaplan-Meier analysis was performed on all patients, no association was shown between expression of MRP-1 at diagnosis (presence/absence, Fig. 2*a,b*) or <25% *vs.* ≥25% expression (Fig. 3*a,b*) and either RFS or OS. Multivariate analysis of all patients revealed the most significant prognostic parameters for RFS to be negative LN status (p = 0.001), positive ER status (p = 0.031) and low histologic grade (p = 0.018). The most significant prognostic factors for OS were negative LN status (p < 0.0001), positive ER status (p = 0.002) and low histologic grade (p = 0.006) (Table II).

Patients were stratified into various subgroups for more detailed analyses (patients who subsequently received chemotherapy, patients who did not receive chemotherapy, node-positive patients, node-negative patients, patients with grade I–III tumours). Kaplan-Meier survival analysis revealed a significant association between ≥25% of tumour cells expressing MRP-1 at diagnosis and worse RFS (p = 0.0181) and OS (p = 0.0171) in patients with grade III tumours treated with adjuvant chemotherapy (Fig. 3*c,d*). Multivariate Cox regression analysis revealed the most significant prognostic factors for RFS in patients with grade III tumours treated with chemotherapy (44 patients) to be negative LN status (p < 0.0001), positive ER status (p = 0.007) and MRP-1 expression in

TABLE II – MULTIVARIATE COX REGRESSION ANALYSIS (BACKWARD STEPWISE, LIKELIHOOD RATIO) IN ALL INVASIVE CARCINOMA PATIENTS, SHOWING *SIGNIFICANT* PARAMETERS[1]

Characteristic	RFS	OS
Histologic grade (I, II *vs.* III)	0.018	0.006
ER status (negative *vs.* positive)	0.031	0.002
Lymph node status (negative *vs.* positive)	0.001	<0.0001

[1]Other parameters included histologic tumour type, age, chemotherapy, tamoxifen treatment, tumour size, MDR-1 Pgp expression (presence/absence and <25% *vs.* ≥25% of tumour cells showing positivity) and MRP-1 protein expression (presence/absence and <25% *vs.* ≥25% cells showing positivity).

<25% of tumour cells at diagnosis ($p = 0.008$). The most significant prognostic factors for OS in this patient group were negative LN status ($p =< 0.0001$), positive ER status ($p = 0.001$) and MRP-1 expression in <25% of tumour cells at diagnosis ($p = .008$) (Table III).

MDR-1 Pgp expression and relation to clinicopathologic features. Using χ^2 analysis, MDR-1 Pgp expression at diagnosis was not significantly associated with tumour size ($p = 0.191$), histologic subtype ($p = 0.711$), LN status ($p = 0.706$), ER status ($p = 0.787$), tamoxifen treatment ($p = 0.526$) or adjuvant chemotherapy treatment ($p = 0.556$). MDR-1 Pgp expression was strongly associated with higher-grade (grade III) tumours; this observation, however, was not statistically significant ($p = 0.085$).

MDR-1 Pgp expression in relation to RFS and OS. Kaplan-Meier analysis of all patients did not reveal any association between MDR-1 Pgp expression at diagnosis (presence/absence and <25% *vs.* ≥25% expression) and either RFS or OS. As in the case of MRP-1 expression, patients were further stratified into various subgroups for analysis (patients who subsequently received chemotherapy, patients who did not receive chemotherapy, node-positive patients, node-negative patients, patients with grade I–III tumours). Representative Kaplan-Meier survival curves can be seen in Figures 4 and 5. No significant associations were shown in any of the subgroups subjected to Kaplan-Meier survival analysis. Survival curves for patients with grade III tumours who received chemotherapy are presented in Figures 4 and 5, where no statistically significant associations are shown between MDR-1 Pgp expression (presence/absence and <25% *vs.* ≥25% expression) and either RFS or OS.

DISCUSSION

In breast cancer, the most frequently used prognostic factors remain those determined by clinical or standard pathologic approaches, namely, LN status, tumour size, histologic grade, nuclear grade and tumour histology. To date, the only valuable predictive factors in this disease are ER and PR status, which can predict response to hormonal treatments. Despite all of the studies directed at identifying new molecular markers of both prognosis and chemosensitivity, only HER-2 appears to hold any promise as a prognostic and possible predictive marker in patients treated with the anti-HER-2 MAb herceptin.[23] Treatment of this malignancy by adjuvant chemotherapy following surgery has increased survival. Treatment regimes include CMF and doxorubicin (Adriamycin), docetaxel (Taxotere), paclitaxel, platinum and more recently herceptin. The effectiveness of chemotherapy is limited by development of MDR. To further define the role of MDR-associated proteins in this disease, expression of the drug transporter proteins MRP-1 and MDR-1 Pgp were investigated in a large series of invasive breast carcinomas.

In our study, MRP-1 protein was expressed in 66.1% of tumours analysed. This observation is in general agreement with reports that MRP-1 is expressed by a considerable number of untreated breast cancers (on average 49.3%).[13] Reported expression patterns have ranged from 20–40%[16,17,24] to 80–100%[24–26] MRP-1 positivity. Conflicting results from several studies regarding the significance of MRP-1 (as well as MDR-1 Pgp) expression are probably explained by a lack of standardisation of the methodology employed.[12,13] Membranous and granular MRP-1 positivity was observed in the majority of tumours studied here, in agreement with previous reports (Fig. 1*c–e*).[26] MRP-1 expression at diagnosis did not correlate with established clinical or pathologic characteristics, namely, ER status, LN status, histologic subtype, histologic grade, tumour size or subsequent treatment with adjuvant chemotherapy. Previous studies also reported that expression of this protein at diagnosis is independent of nodal status, menopausal status, histologic subtype and age.[15,17] Some reports have associated MRP-1 expression with intermediate histologic tumour grade and large tumour size,[15,28] while others have suggested that MRP-1 expression is independent of these parameters.[16] No such correlation was observed in our analysis. We conclude that MRP-1 expression at diagnosis is not necessarily associated with a more aggressive phenotype (and related poorer outcome) in these patients. However, we did observe a significant association between MRP-1 expression at diagnosis and subsequent tamoxifen treatment ($p = 0.033$). ER-negative and PR-negative tumours had less frequent MRP-1 expression in one study;[26] however, we observed no association between MRP-1 expression and ER status.

Studies to date suggest that MRP-1 expression is probably associated with worse prognosis/outcome (reviewed by Leonessa and Clarke[13]). An inverse correlation has been shown between RFS/OS and MRP-1 protein expression in 2 previous studies.[16,26] Using Kaplan-Meier survival analysis, we observed no association between MRP-1 expression at diagnosis (presence/absence and <25% *vs.* ≥25% expression) and either RFS or OS in all of the patients studied. Patients were then stratified into various subgroups for more detailed analyses [patients who subsequently received chemotherapy, patients who did not receive chemotherapy, patients with grade I *vs.* II *vs.* III tumours, LN-positive patients (stage 2) *vs.* LN-negative patients (stage 1), patients with tumours <2 cm *vs.* >2 cm]. No significant correlation between MRP-1 expression and either RFS or OS was found in any of these subgroups with the exception of patients who would be considered to have a less favourable outcome, *i.e.,* stage 1 or stage 2 patients with high-grade tumours (grade III) who were treated with chemotherapy (10 stage 1 patients and 34 stage 2 patients) (Fig. 3). In these patients, a significant association was shown between <25% of tumour cells expressing MRP-1 at diagnosis and increased RFS ($p = 0.0181$) and OS ($p = 0.0171$). When this subgroup was subjected to multivariate analysis, MRP-1 expression in <25% of tumour cells at diagnosis was identified as an independent prognostic factor for both RFS ($p = 0.008$) and OS ($p = 0.008$) (Table III). Nooter *et al.*[16] showed that MRP-1 expression was associated with increased risk of relapse in subgroups of patients (those with small tumours, node-negative patients and node-positive patients treated with CMF). Our results indicate that evaluation of MRP-1 status at diagnosis may provide important prognostic information in subsets of patients considered to have a poor outcome (those with either stage 1 or stage 2 tumours which are high grade and are treated with adjuvant CMF following surgery). Low-level MRP-1 expression may identify patients who will have improved RFS and OS and who should receive perhaps less aggressive chemotherapy. As the number of patients studied here was relatively small, further studies should be carried out on larger numbers of patients with grade III tumours treated with CMF-based chemotherapy following surgery. The relevance of MRP-1 expression in patients treated with other adjuvant therapies that are now being used to treat breast cancer (anthracyclines, taxoids, aromatase inhibitors, Herceptin; reviewed by Kelleher and Miles[29]) should also be investigated.

Patients with grade III tumours treated with adjuvant chemotherapy received either CMF alone or CMF with doxorubicin or paclitaxel. The substrate specificities of MRP-1 suggest a contribution to doxorubicin sensitivity but no effect on CMF (except

TABLE III – MULTIVARIATE COX REGRESSION ANALYSIS (BACKWARD STEPWISE, LIKELIHOOD RATIO) IN CHEMOTHERAPY-TREATED PATIENTS WITH GRADE III TUMOURS, SHOWING *SIGNIFICANT* PARAMETERS[1]

Characteristic	RFS	OS
MRP-1 expression (<25% *vs.* ≥25% positivity)	0.008	0.008
ER status (negative *vs.* positive)	0.007	0.001
LN status (negative *vs.* positive)	<0.0001	<0.0001

[1]Other parameters included histologic tumour type, age (<50 *vs.* ≥50 years at diagnosis), chemotherapy, tamoxifen treatment, tumour size, MDR-1 Pgp expression (presence/absence and <25% *vs.* ≥25% of tumour cells showing positivity) and presence/absence of MRP-1 expression.

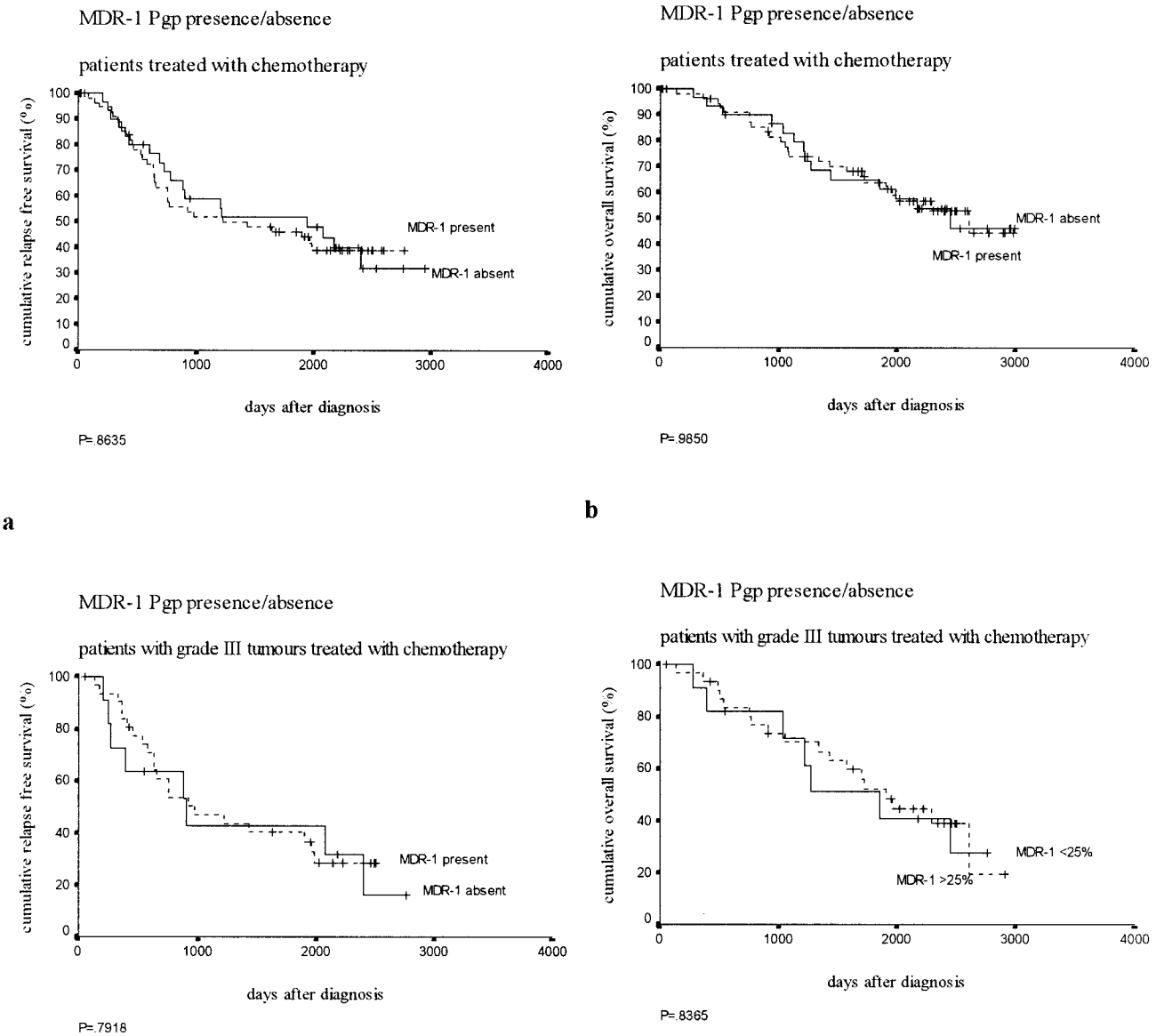

FIGURE 4 – Kaplan-Meier survival analysis for presence/ absence of MDR-1 Pgp in all patients who received chemotherapy *(a,b)* and in patients with grade III tumours treated with chemotherapy *(c,d)* for RFS and OS, respectively. No association can be seen between presence/absence of MDR-1 Pgp at diagnosis and either RFS or OS in either group (p = log rank value).

possibly on methotrexate).[29] MRP-1 may act as a surrogate marker for other resistance-related proteins; or in association with other molecules *in vivo,* its substrate specificity may be altered. Further studies thus should also investigate the contribution to clinical resistance in breast cancer of some of the newly described members of the MRP family and the ABC half-transporter resistance protein (BCRP).[8–11,31]

We observed a highly significant association between MDR-1 Pgp expression and MRP-1 expression at diagnosis in all patients ($p < 0.0001$) and in those subsequently treated with adjuvant chemotherapy ($p = 0.001$). In patients with grade III tumours treated with adjuvant chemotherapy, no correlation was shown between expression of these 2 proteins ($p = 0.815$). Previous studies failed to find a correlation between these 2 proteins,[15] although prechemotherapy MDR-1 Pgp and pre- and postchemotherapy MRP-1 expression predicted tumour recurrence and death in one study.[32] As discussed, MDR-1 Pgp expression in breast cancer has been extensively investigated. Pgp has been associated with poor outcome in both primary and advanced breast cancers.[12] MDR-1 Pgp was expressed at diagnosis in 66% of invasive breast cancers studied here (Fig. 1*a,b*). Several studies have reported expression levels of MDR-1 Pgp varying 0–100% in untreated breast cancers. When data from all studies involving more than 20 patients are pooled, the average rate of MDR-1 Pgp detection is 45.6%.[13] Our results indicate a trend towards MDR-1 Pgp expression being associated with higher-grade tumours (grade III, $p = 0.085$) compared to grade I/II tumours. Previous results regarding such an association have not proved consistent; 2 immunohistochemical studies did not find an association.[14,33] There was no correlation, however, with any of the other clinicopathologic fea-

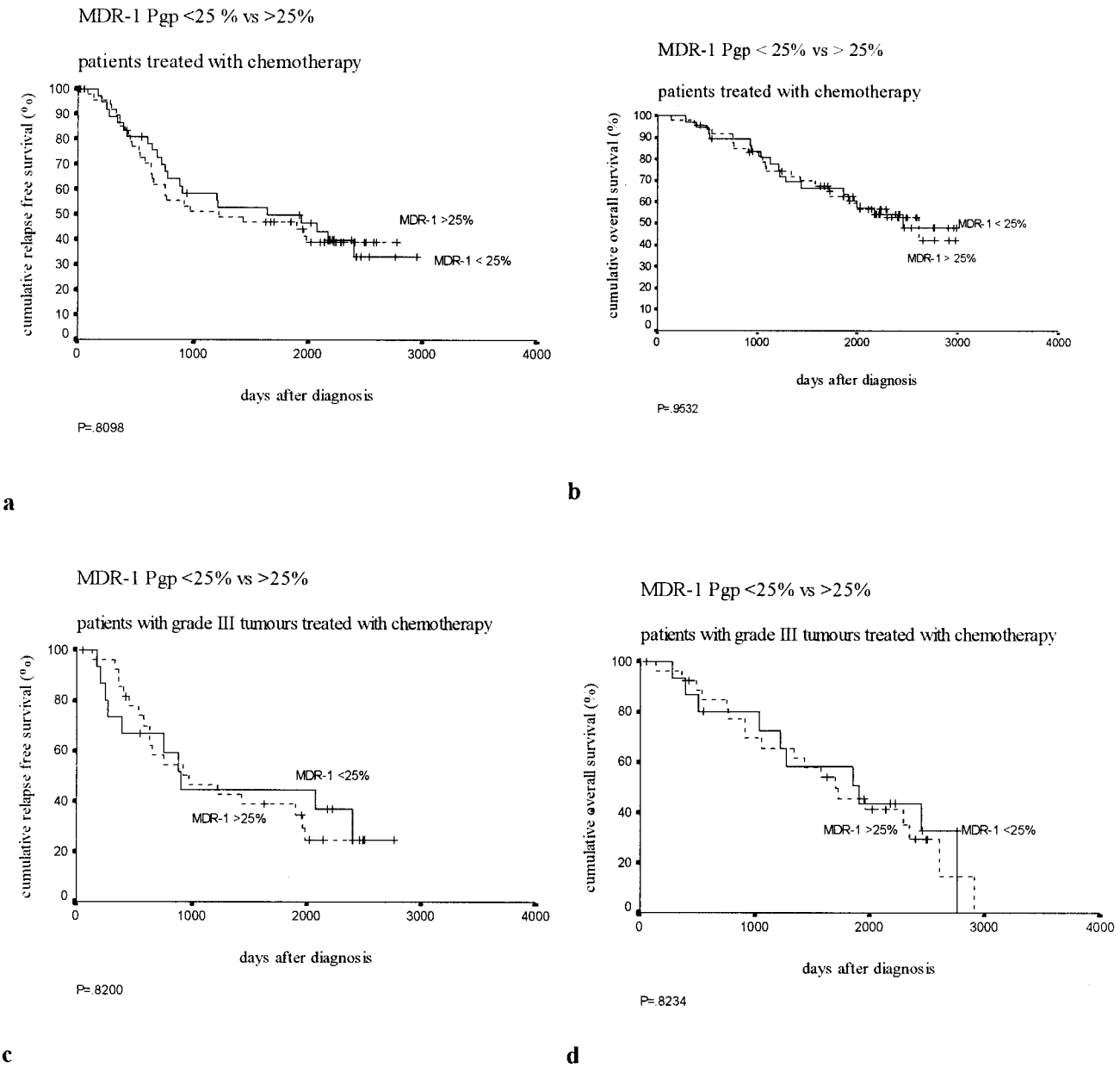

FIGURE 5 – Kaplan-Meier survival analysis for MDR-1 Pgp expression (<25% *vs.* ≥25% tumour cells staining positive) in all patients who received chemotherapy *(a,b)* and in patients with grade III tumours treated with chemotherapy *(c,d)* for RFS and OS, respectively. No association can be seen between <25% *vs.* ≥25% MDR-1 Pgp at diagnosis and either RFS or OS in either group (p = log rank value).

tures studied. Again, most previous studies, in agreement with our findings, have indicated that none of the known clinicopathologic features is significantly associated with expression of MDR-1 Pgp (reviewed by Leonessa and Clarke[13]).

Kaplan-Meier survival analysis of all patients failed to show any significant association between MDR-1 Pgp expression at diagnosis (presence/absence and <25% *vs.* ≥25% expression) and either RFS or OS (Figs. 4, 5). When patients, as in the case of MRP-1 analysis, were stratified according to chemotherapy status, LN status, histologic grade and tumour size, there were no significant associations between MDR-1 Pgp expression and either RFS or OS. Previous results regarding expression of this protein and possible association with RFS or OS have proved conflicting.[13] Our results indicate that MDR-1 Pgp expression at diagnosis does not provide prognostic information in patients with invasive breast carcinoma. Although expression of MRP-1 and MDR-1 Pgp was significantly correlated in the analysis of all patients, no correlation was shown in patients with grade III tumours who received adjuvant chemotherapy, where low-level MRP-1 expression played a prognostic role.

In summary, MRP-1 expression in <25% of tumour cells at diagnosis appears to be a significant marker of improved outcome in a subset of invasive breast cancer patients with a less favourable outcome, *i.e.,* those with high-grade stage 1 or stage 2 cancers who are treated with adjuvant CMF-based chemotherapy following surgery. The question as to whether such expression holds predictive power in these patients requires further investigation. Furthermore, any future studies should also address the prognostic role of other members of the MRP family in the various subgroups of patients with invasive breast carcinoma studied here.

REFERENCES

1. Aapro M. Adjuvant therapy of primary breast cancer: a review of key findings from the 7th International Conference, St. Gallen, February 2001. Oncologist 2001;6:376–85.
2. Glen H, Jones R. 8th International Conference: Primary therapy of early breast cancer, St Gallen, Switzerland, March 12–15, 2003. Breast Cancer Res 2003;5:198–201.
3. Jemel A, Thomas A, Murray T, Thurn M. Cancer statistics. CA Cancer J Clin 2002;52:23–47.
4. Henderson IC, Harris JR, Kinne DW, Hellman S. Cancer of the breast. In: Devita VT, Hellman S, Rosenberg SA, eds. Cancer, principles and practice of oncology. Philadelphia: Lippincott, 1989. 1197–268.
5. Clynes M. Multiple drug resistance in cancer. Cellular, molecular and clinical approaches. Dordrecht: Kluwer, 1993.
6. Higgins CF, Grottesman MM. Is the multidrug transporter a flippase? Trends Biochem Sci 1992;17:18–21.
7. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 1992;258:1605–54.
8. Kool M, De Haas M, Scheffer GL, Scheper RJ, Van Eijk MJT, Juijn, JA, Baas F, Borst P. Analysis of expression of CMOAT (MRP2), MRP3, MRP4 and MRP5 homologues of the multidrug resistance associated protein gene (mrp-1) in human cancer cell lines. Cancer Res 1997;57:3537–47.
9. Kool M, Van der Linden M, Der Haas F, Borst P. Expression of MRP6 a homologue of the multidrug associated protein MRP1 in tissues and cancer cells. Cancer Res 1999;59:175–82.
10. Bera TK, Lee S, Salvatore G, Lee B, Pastan IH. *Mrp8*, a new member of abc transporter superfamily, identified by est database mining and gene prediction program, is highly expressed in breast cancer. Mol Med 2001;7:509–16.
11. Hopper E, Belinsky MG, Zeng H, Tosolini A, Testa JR, Kruh GD. Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. Cancer Lett 2001;162:181–91.
12. Trock BJ, Leonessa F, Clarke R. Multidrug resistance in breast cancer: a meta analysis of mdr-1/gp170 expression and its possible functional significance. J Natl Cancer Inst 1997;89:917–31.
13. Leonessa F, Clarke R. ATP binding cassette transporters and drug resistance in breast cancer. Endocr Relat Cancer 2003;10:43–73.
14. Chevillard S, Pouillart P, Beldjord C, Asselain B, Beuzeboc P, Magdelenat H, Vielh P. Sequential assessment of multidrug resistance phenotype and measurement of S-phase fraction as predictive markers of breast cancer response to neoadjuvant chemotherapy. Cancer 1996; 77:292–300.
15. Filipits M, Malayeri R, Suchomel RW, Stranzi T, Dekan G, Kaider A, Stiglbauer W, Depisch D, Pirker R. Expression of the multidrug resistance protein (MRP1) in breast cancer. Anticancer Res 1999;19: 5043–9.
16. Nooter K, de la Riviere GB, Klijn J, Stoter G, Foekens J. Multidrug resistance protein in recurrent breast cancer. Lancet 1997;349: 1885–6.
17. Nooter K, de la Riviere GB, Look MP, van Wingerden KE, Henzen-Logmans SC, Scheper RJ, Flens MJ, Klijn JGM, Stoter G, Foekens JA. The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer. Br J Cancer 1997;76:486–93.
18. Page DL, Anderson AT. Diagnostic pathology. London: Churchill Livingstone, 1987.
19. Elston CW, Ellis IO. Pathologic prognostic factors in breast cancer. I. The value of histologic grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
20. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) immunoperoxidase techniques. J Histochem 1981;29:577–80.
21. Moran E, Larkin AM, Doherty G, Kelehan P, Kennedy SM, Clynes M. A new *mdr-1* encoded P-170 specific monoclonal antibody: (6/1C) on paraffin wax embedded tissue without pretreatment of sections. J Clin Pathol 1997;50:465–71.
22. Flens MJ, Izquierdo MA, Scheffer GL, Fritz JM, Meijer JLM, Scheper RJ, Zaman GJR. Immunocytochemical detection of the multidrug resistance associated protein MRP in human multidrug resistant tumour cells by monoclonal antibodies. Cancer Res 1994;54:4557–63.
23. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344:783–92.
24. Linn SC, Pinedo HM, van Ark-Otte J, van der Valk P, Hoekman K, Honkoop AH, Vermorken JB, Giaccone G. Expression of drug resistance proteins in breast cancer, in relation to chemotherapy. Int J Cancer 1997;71:787–95.
25. Dexter DW, Reddy RK, Geles KG, Bansal S, Myint MA, Rogakto A, Leighton JC, Goldstein LJ. Quantitative reverse transcriptase-polymerase chain reaction measured expression of *mdr* and *mrp* in primary breast carcinoma. Clin Cancer Res 1998;4:1533–42.
26. Filipits M, Suchomel RW, Dekan G, Haider K, Valdimarsson G, Depisch D, Pirker R. *mrp* and *mdr-1* gene expression in primary breast carcinomas. Clin Cancer Res 1996;2:1231–7.
27. Flens MJ, Zaman GJR, van der Valk P, Izquierdo MA, Schroeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJLM, Scheper RJ. Tissue distribution of the multidrug resistance protein. Am J Pathol 1996;148:1237–47.
28. Beck J, Bohnet B, Brugger D, Bader P, Dietl J, Scheper RJ, Kandolf R, Liu C, Niethammer D, Gekeler V. Multiple gene expression analysis reveals distinct differences between G2 and G3 stage breast cancers, and correlations of PKCn with MDR1, MRP and LRP gene expression. Br J Cancer 1998;77:87–91.
29. Kelleher M, Miles D. The adjuvant treatment of breast cancer. Int J Clin Pract 2003;57:195–9.
30. Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G. Antifolate resistance mediated by the multidrug resistance proteins MRP-1 and MRP-2. Cancer Res 1999;59:2532–5.
31. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA 1998;95:15665–70.
32. Mechetner E, Kyshtoobayeva A, Zonis S, Kim H, Stroup R, Garcia R, Parker RJ, Fruehauf JP. Levels of multidrug resistance (MDR-1) P-glycoprotein expression by human breast cancer correlate with in vitro resistance to Taxol and doxorubicin. Clin Cancer Res 1998;4: 389–98.
33. Charpin C, Vielh P, Duffaud F, Devictor B, Lavaut MN, Allasia C, Horscowski N, Piana L. Quantitative immunocytochemical assays of P-glycoprotein in breast carcinomas: correlation to messenger RNA expression and to immunohistochemical prognostic indicators. J Natl Cancer Inst 1994;86:1539–45.